Experimental

Cell Growth and Protein Purification. The mutant DJ495 strain of Azotobacter vinelandii was grown in a 24-L fermentor at 30°C in a modified, liquid Burk's medium . Nitrogenase derepression and cell-extract preparation were preformed as previously described [1]. Nitrogenase component proteins were separated by anaerobic Q-Sepharose anion-exchange column chromatography using a linear NaCl concentration gradient. The Fe protein was purified to homogeneity by fractionation from a second Q-Sepharose column. The α-H195Q MoFe protein was further purified by Sephacryl S-200 gel filtration before Phenyl-Sepharose hydrophobic-interaction chromatography as previously described [2]. Purified α-H195Q MoFe protein and Fe protein had specific activities of 2000-2800 nmol of H_2 (min/mg protein)$^{-1}$ and 2000-2300 nmol of H_2 (min/mg protein)$^{-1}$ respectively. The purified component proteins were exchanged into D_2O buffer containing 25 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 250 mM NaCl. Protein concentrations were determined by the Lowry method and metal content was determined by ICP spectroscopy using a Perkins-Elmer Plasma 400 spectrometer. Hydrogen evolution was measured on a Shimadzu GC-8A gas chromatograph fitted with a thermal conductivity detector.

Preparation of CO-Inhibited Nitrogenase. Each sample was prepared in a 15-mL reaction vial containing 30 mg total protein in 2.25-mL total volume as previously described [2] with the following changes. Low electron-flux conditions were obtained using a 1:4 molar ratio of Fe protein:MoFe protein. The reaction-mixture components, which consisted of 2.5 mM ATP, 5 mM $MgCl_2$, 30 mM creatine phosphate, 25 units/mL of creatine phosphokinase in 25 mM HEPES, pH 7.4 and 20 mM sodium dithionite were prepared in D_2O. Turnover was accomplished under 101 kPa CO (either $^{12}C^{16}O$, $^{13}C^{16}O$, $^{13}C^{18}O$ or an appropriate mixture). The reaction was quenched by the addition of ethylene glycol to a final concentration of 40% after 15 minutes. The resulting turnover product was concentrated in an Amicon microfiltration pressure concentrator using a regenerated cellulose PLHK ultrafiltration membrane with a 100,000 molecular weight cut off under 202 kPa CO of the same composition used for the turnover reaction.

Photolysis and FT-IR spectroscopy. The sample photolysis was conducted in an Oxford cryostat with CaF_2 external windows, ZnSe intermediate windows, and ZrO_2 windows for the IR cell along the IR pathway. Spectra were recorded at 4 cm^{-1} resolution with a Bruker V-70v FT-IR spectrometer using a MCT detector. For photolysis, a Sutter Instruments 300 W Lambda LS xenon arc lamp was shone through the side of the cryostat oriented 90° to the IR light path. The sample was held at 45° to both beams. This allowed the use of quartz windows for the visible light. The estimated power on the sample was as high as 1 W.

Wavelength Dependent Photolysis. We used a set of VersaChrome® tunable bandpass filters (Semrock). By adjusting the incident angle, the central wavelength is tunable with

FWHM bandwidth as 20 nm. The central wavelengths we used are 340, 380, 410, 440, 470, 510, 550, 610, 700 and 800 nm.

Experimental References:

[1] **D. J. Scott, D. R. Dean, W. E. Newton, *J. Biol. Chem.* 1992, *267*, 20002-20010.**

[2] **C.-H. Kim, W. E. Newton, D. R. Dean, *Biochemistry* 1995, *34*, 2798-2808.**

DFT

Methodology Details. All the DFT calculations were done using the PBE (1) functional and the LACV3P** basis set, as implemented in JAGUAR 7.7 (2) software. For the first- and second-row elements, LACV3P** implies a 6-311G** triple-zeta basis set including polarization function. For the Fe and Mo atoms, LACV3P** uses the Los Alamos effective core potential (ECP), and the valence part is essentially of triple-zeta quality. The geometries optimized at the PBE/LACV3P** level were used for the analytic Hessian calculations, resulting in the harmonic frequencies and IR intensities discussed in the text. We found the present and essentially equivalent setups to perform well for the vibrational dynamics of the iron-sulfur systems (3-5). For a methodology comparable to ours, ~1% underestimation of the observed vibrational frequencies by calculation is not uncommon (6, 7). The presently calculated frequencies were scaled by +0.4%, corresponding to the 2143/2135 ≈ 1.004 ratio of the observed/calculated (PBE/LACV3P**) gas-phase $\nu(C\equiv O)$ frequencies. Translated to the absolute frequencies of the FeMo-co Hi-3 and Lo-3 photolytic species, this scaling implies virtually uniform ~8 cm^{-1} positive shift. The analysis of the computed vibrational normal modes has been facilitated using an in-house Q-SPECTOR Python tool, applied to model the FT-IR spectra and assess the FeMo-co-bound CO modes via kinetic energy distribution (KED) approach. The 5 cm^{-1} Lorentzian broadening of the calculated mode intensities was empirically found to fit well the FT-IR data, recorded at 4 cm^{-1} resolution.

Structural Modelling. Initial coordinates for the cofactor iron-sulfur [Mo-7Fe-9S-**X**] core and its covalent ligands Cys275, His442, R-homocitrate (HCA) were extracted from the 1.16 Å resolution 1M1N PDB file (8). Cys275 was simplified to methylthiolate, His442 to imidazole, and HCA to glycolate ($^{-}OCH_2$-COO^{-}).

Metal Sites Oxidation Level and Spin Coupling. The present modelling assumes [$Mo^{4+}2Fe^{3+}5Fe^{2+}$] formal oxidation levels for the transition metal ions entering FeMo-co, and the total spin of $S = 0$. This oxidation level corresponds to the cofactor $1e^-$ reduced, as compared to the $[Mo^{4+}3Fe^{3+}4Fe^{2+}]$ ($S = 3/2$) resting state, as proposed earlier (9-12). A set of seven Fe sites spin vectors satisfying the total FeMo-cofactor $S = 0$ spin is not unique; as earlier for the $2e^-$ state (13), we used BS2 spin-collinear ("up" ↑ or "down" ↓) coupling (14) for Fe sites following the broken symmetry (BS) concept (15). The initial BS2 electronic structure was constructed using an option to assign a number of unpaired α/β electrons and formal charges to Fe atomic fragments, as implemented in JAGUAR 7.7. The resulting self-consisted field (SCF) solution bears a mixed-valence Fe^{3+}/Fe^{2+} character, however retaining the spin densities BS2 pattern.

Table S1. Simulation of the mixed isotope experiment. The ~25% $^{12}C^{16}O$ / ~75% $^{13}C^{18}O$ mixed isotope Hi-3→Lo-3 photolysis spectra (actual and the hypothetical 'decoupled' model, see the text) were simulated using the DFT calculated ν(C≡O) frequencies and their IR intensities for the following isotope alternatives bound at Fe2 *exo* and Fe6 *exo*, with their weights specified:

Fe2/Fe6-bound CO isotope	Actual model simulation weight	'Decoupled' model simulation weight
$^{12}C^{16}O/^{12}C^{16}O$	-1/16	-1/4
$^{12}C^{16}O$/None (Lo-3)¼	1/4	1/4
$^{12}C^{16}O/^{13}C^{18}O$	-3/16	0
$^{13}C^{18}O/^{12}C^{16}O$	-3/16	0
$^{13}C^{18}O/^{13}C^{18}O$	-9/16	-3/4
$^{13}C^{18}O$/None (Lo-3)	3/4	3/4

Table S2. Hi-3/Lo-3 pure and mixed $^{12}C^{16}O/^{13}C^{18}O$ isotope analysis using FT-IR and DFT results for **X** = N^{3-} modelling. For the DFT broadened peak relative intensities corresponding to double difference spectra modelling, see Figure 5.

	Symmetric/Asymmetric Hi-3 *or* Lo-3 ν(C≡O) Bands by FT-IR		Symmetric/Asymmetric Hi-3 *or* Lo-3 ν(C≡O) Modes by DFT		
Fe2/Fe6-bound CO isotope	Band Frequency, cm^{-1}	Band Strength, Relative	Frequency, cm^{-1}	IR Intensity, Relative (Absolute, km/mol)	Fe2-bound CO KED[c]
$^{12}C^{16}O/^{12}C^{16}O$	1938/1911 (Hi-3)	3.7/1[a]	1938/1909	3.2/1[a] (1958/605)	0.83/0.17
$^{12}C^{16}O$/None	1921 (Lo-3)	1.7[a]	1923	2.3[a] (1372)	1.00
$^{12}C^{16}O/^{13}C^{18}O$	1934/-[b]	-/-	1934/1825	1.5/1 (1482/977)	0.99/0.01
$^{13}C^{18}O/^{12}C^{16}O$	1917/-[b]	-/-	1916/1842	1.5/1 (1480/962)	0.02/0.98
$^{13}C^{18}O/^{13}C^{18}O$	1850/1824	4.9/1[a]	1848/1821	3.3/1[a] (1788/548)	0.82/0.18
$^{13}C^{18}O$/None	1833	2.7[a]	1834	2.3[a] (1250)	1.00

[a]) Pure isotope Hi-3/Lo-3 IR strengths are given relatively to the lower frequency asymmetric Hi-3 mode.

[b]) This mixed isotope band could not be resolved by the experiment.

[c]) The kinetic energy distribution (KED) values from DFT describe the fraction of the vibrational mode energy accumulated in the Fe2-bound C≡O stretching mode.

Table S3. Hi-3/Lo-3 pure and mixed $^{12}C^{16}O/^{13}C^{18}O$ isotope analysis using FT-IR and DFT results for **X** = C^{4-} modelling.

	Symmetric/Asymmetric Hi-3 *or* Lo-3 ν(C≡O) Bands by FT-IR		Symmetric/Asymmetric Hi-3 *or* Lo-3 ν(C≡O) Modes by DFT		
Fe2/Fe6-bound CO isotope	Band Frequency, cm^{-1}	Band Strength, Relative	Frequency, cm^{-1}	IR Intensity, Relative (Absolute, km/mol)	Fe2-bound CO KED[c]
$^{12}C^{16}O/^{12}C^{16}O$	1938/1911 (Hi-3)	3.7/1[a]	1932/1903	3.6/1[a] (1803/501)	0.77/0.23
$^{12}C^{16}O$/None	1921 (Lo-3)	1.7[a]	1924	2.4[a] (1195)	1.00
$^{12}C^{16}O/^{13}C^{18}O$	1934/-[b]	-/-	1927/1820	1.5/1 (1308/898)	0.98/0.01
$^{13}C^{18}O/^{12}C^{16}O$	1917/-[b]	-/-	1911/1834	1.7/1 (1387/808)	0.02/0.97
$^{13}C^{18}O/^{13}C^{18}O$	1850/1824	4.9/1[a]	1842/1815	3.6/1[a] (1644/454)	0.76/0.23
$^{13}C^{18}O$/None	1833	2.7[a]	1835	2.4[a] (1088)	1.00

[a]) Pure isotope Hi-3/Lo-3 IR strengths are given relatively to the lower frequency asymmetric Hi-3 mode.

[b]) This mixed isotope band could not be resolved by the experiment.

[c]) The kinetic energy distribution (KED) values from DFT describe the fraction of the vibrational mode energy accumulated in the Fe2-bound C≡O stretching mode.

DFT-optimized coordinates (in XYZ format) for the Hi-3 (Fe2 *exo*, Fe6 *exo*) X = N^{3-} model:

Mo	0.986047	-3.318144	-0.373594
Fe	0.588447	3.713270	0.652686
Fe	-0.862256	1.318057	1.105905
Fe	1.850034	1.422048	0.997921
Fe	0.356102	1.765619	-1.236233
Fe	0.557729	-0.852738	-1.483260
Fe	-0.799709	-1.439530	0.615670
Fe	1.957949	-1.025981	0.591807
S	-1.288939	3.168076	-0.397575
S	0.607323	2.389032	2.586836
S	0.207915	0.624996	-3.137410
S	2.288921	2.970653	-0.669757
S	3.526920	0.149993	1.616913
S	-1.011668	-2.525772	-1.347584
S	-2.426854	0.025608	-0.026380
S	0.841207	-2.510725	1.861670
S	2.634992	-1.907624	-1.403250
S	0.911370	5.899284	1.284144
C	2.538078	5.709246	2.136343
C	0.531290	-5.698355	-2.494729
C	0.717942	-5.911024	1.010780
C	2.235519	-5.815759	0.750686
C	1.917838	-4.359020	-3.500279
C	1.685272	-5.395061	-4.375108
H	2.055991	-5.606758	-5.373138
H	-0.123365	-6.085766	-1.713325
H	0.414751	-7.105881	-4.075701
H	0.419809	-6.979024	0.919104
H	0.569396	-5.625331	2.080749
H	3.303096	5.360679	1.426660
H	2.845393	6.681611	2.558581
H	2.460470	4.970511	2.947861
H	2.540420	-3.474288	-3.594848
N	0.799445	-6.239836	-3.717419
N	0.513498	0.223819	0.147531
N	1.198394	-4.565819	-2.345633
O	3.014187	-6.718717	1.091584
O	2.575310	-4.701449	0.158794
O	-0.019985	-5.092595	0.153659
O	-2.951241	1.942477	3.059593
C	-2.113056	1.704228	2.277168
O	-2.658868	-2.655882	2.493125
C	-1.904980	-2.148681	1.746973
H	-1.944347	0.289417	-1.285457

DFT-optimized coordinates (in XYZ format) for the Lo-3 (Fe2 *exo*) X = N^{3-} model:

Mo	0.908019	-3.362470	-0.308102
Fe	0.632059	3.748947	0.470580
Fe	-0.790129	1.403514	1.152174
Fe	1.977192	1.486983	0.915884
Fe	0.432462	1.712805	-1.257587
Fe	0.668206	-0.892607	-1.516801
Fe	-0.614233	-1.317142	0.559494
Fe	1.865208	-1.048078	0.679425
S	-1.303533	3.056198	-0.484775
S	0.746947	2.582535	2.470798
S	0.411988	0.528853	-3.183642
S	2.355810	2.978359	-0.806583
S	3.479345	0.016893	1.682318
S	-1.020976	-2.484842	-1.377674
S	-2.545821	-0.060932	0.474134
S	0.710152	-2.578480	1.938668
S	2.679746	-2.007385	-1.205172
S	0.814445	5.958847	0.923924
C	2.186417	6.026141	2.158966
C	0.462772	-5.660602	-2.519288
C	0.483721	-5.960130	1.016708
C	2.011277	-5.927631	0.810691
C	1.793662	-4.245571	-3.495244
C	1.546415	-5.235981	-4.417824
H	1.880982	-5.382940	-5.439848
H	-0.176717	-6.090694	-1.747996
H	0.312372	-6.981341	-4.170814
H	0.144387	-7.014113	0.908710
H	0.310080	-5.672261	2.082321
H	3.100828	5.586403	1.732108
H	2.388582	7.076199	2.437274
H	1.919383	5.455216	3.061312
H	2.392260	-3.342785	-3.571646
N	0.694949	-6.129153	-3.779149
N	0.517704	0.164622	0.109454
N	1.118180	-4.528086	-2.330088
O	2.739377	-6.861491	1.178196
O	2.415983	-4.829511	0.231018
O	-0.187538	-5.107417	0.137547
O	-2.575053	2.115175	3.362918
C	-1.846999	1.819293	2.493429
H	-2.401804	0.115730	-0.870750

Animated vibrational modes. Normal modes displaying FeMo-cofactor bound C≡O stretching vibrations for the structures involving $^{12}C^{16}O$ isotope bound at Fe2, see Table S2. The files in GIF format are available for download from the journal website.

DFT References:

1. Perdew JP, Burke K, & Ernzerhof M (1996) *Physical Review Letters* 77, 3865-3868.
2. Schrödinger L, New York, NY. (2010) (Schrödinger, LLC, New York, NY.).
3. Pelmenschikov V, Guo YS, Wang HX, Cramer SP, & Case DA (2011) *Faraday Discussions* 148, 409-420.
4. Mitra D, Pelmenschikov V, Guo YS, Case DA, Wang HX, Dong WB, Tan ML, Ichiye T, Jenney FE, Adams MWW*, et al.* (2011) *Biochemistry* 50, 5220-5235.
5. Guo YS, Wang HX, Xiao YM, Vogt S, Thauer RK, Shima S, Volkers PI, Rauchfuss TB, Pelmenschikov V, Case DA*, et al.* (2008) *Inorg. Chem.* 47, 3969-3977.
6. Dance I (2011) *Dalton Trans.* 40, 6480-6489.
7. Merrick JP, Moran D, & Radom L (2007) *Journal of Physical Chemistry A* 111, 11683-11700.
8. Einsle O, Tezcan FA, Andrade SLA, Schmid B, Yoshida M, Howard JB, & Rees DC (2002) *Science* 297, 1696-1700.
9. Dance I (2006) *Inorg. Chem.* 45, 5084-5091.
10. Schimpl J, Petrilli HM, & Blochl PE (2003) *J. Am. Chem. Soc.* 125, 15772-15778.
11. Lovell T, Liu TQ, Case DA, & Noodleman L (2003) *J. Am. Chem. Soc.* 125, 8377-8383.
12. Yoo SJ, Angove HC, Papaefthymiou V, Burgess BK, & Munck E (2000) *J. Am. Chem. Soc.* 122, 4926-4936.
13. Pelmenschikov V, Case DA, & Noodleman L (2008) *Inorg. Chem.* 47, 6162-6172.
14. Lovell T, Li J, Liu TQ, Case DA, & Noodleman L (2001) *J. Am. Chem. Soc.* 123, 12392-12410.
15. Noodleman L (1981) *Journal of Chemical Physics* 74, 5737-5743.